

ANTISENSE MODULATION OF SURVIVIN EXPRESSION**RELATED APPLICATIONS**

5 This application is a continuation-in-part of U.S. Serial No. 09/918,186, filed July 30, 2001, which is a continuation-in-part of U.S. Serial No. 09/496,694, filed February 2, 2000, which is a continuation-in-part of U.S. Serial No. 09/286,407 filed April 5, 1999, issued as U.S. 10 Patent 6,165,788, which is a continuation-in-part of U.S. Serial No. 09/163,162 filed September 29, 1998, issued as U.S. Patent 6,077,709.

FIELD OF THE INVENTION

15 The present invention provides compositions and methods for modulating the expression of Survivin. In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human Survivin. Such oligonucleotides have been 20 shown to modulate the expression of Survivin.

BACKGROUND OF THE INVENTION

25 A hallmark feature of cancerous cells is uncontrolled proliferation. Among the differences that have been discovered between tumor and normal cells is resistance to the process of programmed cell death, also known as apoptosis (Ambrosini et al., *Nat. Med.*, 1997, 3, 917-921). Apoptosis is a process multicellular organisms have evolved to prevent uncontrolled cell proliferation as well as to eliminate cells 30 that have become sick, deleterious, or are no longer necessary. The process of apoptosis involves a multistep cascade in which cells are degraded from within, through the concerted action of proteolytic enzymes and DNA endonucleases, resulting in the formation of apoptotic bodies that are then

removed by scavenger cells. Research to date has shown that much of the intracellular degradation is carried out through the action of the caspases, a family of proteolytic enzymes that cleave adjacent to aspartate residues (Cohen,
5 *Biochemistry Journal*, 1997, 326, 1-16).

The finding that most tumor cells display resistance to the apoptotic process has led to the view that therapeutic strategies aimed at attenuating the resistance of tumor cells to apoptosis could represent a novel means to halt the spread
10 of neoplastic cells (Ambrosini et al., *Nat. Med.*, 1997, 3, 917-921). One of the mechanisms through which tumor cells are believed to acquire resistance to apoptosis is by overexpression of Survivin, a recently described member of the IAP (inhibitor of apoptosis) caspase inhibitor family. To
15 date, overexpression of Survivin has been detected in tumors of the lung, colon, pancreas, prostate, breast, stomach, non-Hodgkin's lymphoma, and neuroblastoma (Adida et al., *Lancet*, 1998, 351, 882-883; Ambrosini et al., *Nat. Med.*, 1997, 3, 917-921; Lu et al., *Cancer Res.*, 1998, 58, 1808-1812). A more
20 detailed analysis has been performed in neuroblastoma where it was found that Survivin overexpression segregated with tumor histologies known to associate with poor prognosis (Adida et al., *Lancet*, 1998, 351, 882-883). Finally, Ambrosini et al. describe transfection of HeLa cells with an expression vector
25 containing a 708 nt fragment of the human cDNA encoding effector cell protease receptor 1 (EPR-1), the coding sequence of which is extensively complementary to the coding strand of Survivin (Ambrosini et al., *J. Bio. Chem.*, 1998, 273, 11177-11182) and which potentially acts as a Survivin antisense RNA.
30 This construct caused a reduction in cell viability. Methods for modulating apoptosis and for reducing the severity of a pathological state mediated by Survivin using agents that modulate amounts or activity of Survivin are disclosed in WO 98/22589, which also discloses the EPR-1 coding

strand/Survivin antisense construct described by Ambrosini et al., *supra*.

Survivin has recently been found to play a role in cell cycle regulation. It has been found to be expressed in the 5 G2/M phase of the cell cycle in a cycle-regulated manner, and associates with microtubules of the mitotic spindle. Disruption of this interaction results in loss of Survivin's anti-apoptotic function and increased caspase-3 activity during mitosis. Caspase-3 is associated with apoptotic cell 10 death. It is therefore believed that Survivin may counteract a default induction of apoptosis in G2/M phase. It is believed that the overexpression of Survivin in cancer may overcome this apoptotic checkpoint, allowing undesired survival and division of cancerous cells. The Survivin antisense construct 15 described by Ambrosini above was found to downregulate endogenous Survivin in HeLa cells and to increase caspase-3-dependent apoptosis in cells in G2/M phase. Li et al., *Nature*, 1998, 396, 580-584.

As a result of these advances in the understanding of 20 apoptosis and the role that Survivin expression is believed to play in conferring a growth advantage to a wide variety of tumor cell types, there is a great desire to provide compositions of matter which can modulate the expression of Survivin. It is greatly desired to provide methods of 25 diagnosis and detection of nucleic acids encoding Survivin in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from Survivin expression. In addition, improved research kits and reagents for detection and study of nucleic acids encoding Survivin are desired.

30 Currently, there are no known therapeutic agents which effectively inhibit the synthesis of Survivin. Consequently, there is a long-felt need for agents capable of effectively inhibiting Survivin expression in tumor cells. Antisense oligonucleotides against Survivin may therefore prove to be

uniquely useful in a number of therapeutic, diagnostic and research applications.

SUMMARY OF THE INVENTION

5 The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding Survivin, and which modulate the expression of Survivin. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also
10 provided. Further provided are methods of modulating the expression of Survivin in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a
15 human, suspected of having or being prone to a disease or condition associated with expression of Survivin by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

20 One embodiment of the present invention is a method of inhibiting the expression of Survivin in human cells or tissues comprising contacting human cells or tissues with an antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so that
25 expression of Survivin is inhibited.

30 The present invention also provides a method of treating an animal having a disease or condition associated with Survivin comprising administering to an animal having a disease or condition associated with Survivin a therapeutically or prophylactically effective amount of an antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so that expression of Survivin is inhibited. Preferably, the disease or condition is a hyperproliferative condition. In one

embodiment, the hyperproliferative condition is cancer

Another embodiment of the present invention is a method of treating a human having a disease or condition characterized by a reduction in apoptosis comprising 5 administering to a human having a disease or condition characterized by a reduction in apoptosis a prophylactically or therapeutically effective amount an of antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so that expression of 10 survivin is inhibited.

The present invention also provides a method of modulating apoptosis in a cell comprising contacting a cell with an antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so 15 that apoptosis is modulated.

Still another embodiment of the invention is a method of modulating cytokinesis in a cell comprising contacting a cell with an antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so 20 that cytokinesis is modulated.

The present invention also provides a method of modulating the cell cycle in a cell comprising contacting a cell with an antisense compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding human survivin so 25 that the cell cycle is modulated.

In still another embodiment of the invention, there is provided a method of inhibiting the proliferation of cells comprising contacting cells with an effective amount of an antisense compound 8 to 80 nucleobases in length targeted to a 30 nucleic acid molecule encoding human survivin, so that proliferation of the cells is inhibited. In one embodiment, the cells are cancer cells. The method may further comprise administering to the patient a chemotherapeutic agent.

Preferably, the modulation of apoptosis is sensitization

to an apoptotic stimulus. In one embodiment, the apoptotic stimulus is a cytotoxic chemotherapeutic agent. The method may further comprise contacting the cells with a chemotherapeutic agent. Preferably, the chemotherapeutic agent is taxol or 5 cisplatin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in 10 modulating the function of nucleic acid molecules encoding Survivin, ultimately modulating the amount of Survivin produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding Survivin. As used herein, the terms 15 "target nucleic acid" and "nucleic acid encoding Survivin" encompass DNA encoding Survivin, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes 20 with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense".

The functions of DNA to be interfered with include 25 replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic 30 activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of Survivin. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in

the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding Survivin. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for

translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate 5 translation of an mRNA molecule transcribed from a gene encoding Survivin, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of 10 three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 15 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either 20 25 direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination 25 codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the 30 translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation

termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly 10 translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-15 exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease.

Aberrant fusion junctions due to rearrangements or 20 deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, 25 oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means 30 hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two

nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are 5 considered to be complementary to each other at that position.

The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to 10 indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense 15 compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of 20 utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the 25 case of *in vitro* assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression 30 with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for

research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic 5 moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can 10 be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term 15 includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native 20 forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species 25 the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is 30 believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*, 1995, 81, 611-

620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95, 15502-15507). The posttranscriptional antisense mechanism 5 defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of 10 endogenous targeted mRNA levels (Fire et al., *Nature*, 1998, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, 2002, 295, 694-697).

15 While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance 20 with this invention preferably comprise from about 8 to about 80 nucleobases. In another embodiment, the oligonucleotide is about 12-50 nucleotides in length. In yet another embodiment, the oligonucleotide is 15 to 30 nucleotides in length. Preferred embodiments comprise at 25 least an 8-nucleobase portion of a sequence of an antisense compound which inhibits expression of Survivin. dsRNA molecules directed to survivin (RNAi), and their use in inhibiting survivin mRNA expression, are also within the scope of the present invention.

30 The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenine, variants may be produced which contain thymidine (or uridine if RNA),

guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted 5 with any of the three alternate nucleotides. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of survivin mRNA.

As is known in the art, a nucleoside is a base-sugar 10 combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate 15 group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric 20 compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the 25 internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Oligomer and Monomer Modifications

As is known in the art, a nucleoside is a base-sugar 30 combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar

portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups 5 covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may 10 have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside linkage or in conjunction with the sugar 15 ring the backbone of the oligonucleotide. The normal internucleoside linkage that makes up the backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages

20 Specific examples of preferred antisense oligomeric compounds useful in this invention include oligonucleotides containing modified e.g. non-naturally occurring internucleoside linkages. As defined in this specification, oligonucleotides having modified 25 internucleoside linkages include internucleoside linkages that retain a phosphorus atom and internucleoside linkages that do not have a phosphorus atom. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus 30 atom in their internucleoside backbone can also be considered to be oligonucleosides.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly interfere with RNAi activity. Based on this

observation, it is suggested that certain preferred oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is 5 the phosphorothioate internucleoside linkage.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphoro-dithioates, phosphotriesters, aminoalkylphosphotriesters, 10 methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, 15 thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides 20 having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms 25 are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 30 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of

which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, oligomeric compounds have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -
5 $\text{CH}_2\text{-NH-O-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2-$ and $-\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2-$ [wherein the native phosphodiester internucleotide linkage is represented as -
10 $\text{O-P(=O)(OH)-O-CH}_2-$]. The MMI type internucleoside linkages are disclosed in the above referenced U.S. patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside
15 linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones;
20 riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.
25

30 Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677;

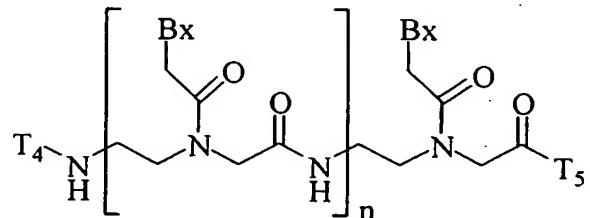
5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;
5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;
5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and
5,677,439, certain of which are commonly owned with this
5 application, and each of which is herein incorporated by
reference.

Oligomer Mimetics

Another preferred group of oligomeric compounds
10 amenable to the present invention includes oligonucleotide
mimetics. The term mimetic as it is applied to
oligonucleotides is intended to include oligomeric
compounds wherein only the furanose ring or both the
furanose ring and the internucleotide linkage are replaced
15 with novel groups, replacement of only the furanose ring is
also referred to in the art as being a sugar surrogate.
The heterocyclic base moiety or a modified heterocyclic
base moiety is maintained for hybridization with an
appropriate target nucleic acid. One such oligomeric
20 compound, an oligonucleotide mimetic that has been shown to
have excellent hybridization properties, is referred to as
a peptide nucleic acid (PNA). In PNA oligomeric compounds,
the sugar-backbone of an oligonucleotide is replaced with
an amide containing backbone, in particular an
25 aminoethylglycine backbone. The nucleobases are retained
and are bound directly or indirectly to aza nitrogen atoms
of the amide portion of the backbone. Representative
United States patents that teach the preparation of PNA
oligomeric compounds include, but are not limited to, U.S.:
30 5,539,082; 5,714,331; and 5,719,262, each of which is
herein incorporated by reference. Further teaching of PNA
oligomeric compounds can be found in Nielsen et al.,
Science, 1991, 254, 1497-1500.

PNA has been modified to incorporate numerous

modifications since the basic PNA structure was first prepared. The basic structure is shown below:



wherein

5 Bx is a heterocyclic base moiety;

 T₄ is hydrogen, an amino protecting group, -C(O)R₅, substituted or unsubstituted C₁-C₁₀ alkyl, substituted or unsubstituted C₂-C₁₀ alkenyl, substituted or unsubstituted C₂-C₁₀ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical 10 functional group, a reporter group, a conjugate group, a D or L α -amino acid linked via the α -carboxyl group or optionally through the ω -carboxyl group when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl 15 group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

 T₅ is -OH, -N(Z₁)Z₂, R₅, D or L α -amino acid linked via 20 the α -amino group or optionally through the ω -amino group when the amino acid is lysine or ornithine or a peptide derived from D, L or mixed D and L amino acids linked through an amino group, a chemical functional group, a reporter group or a conjugate group;

25 Z₁ is hydrogen, C₁-C₆ alkyl, or an amino protecting group;

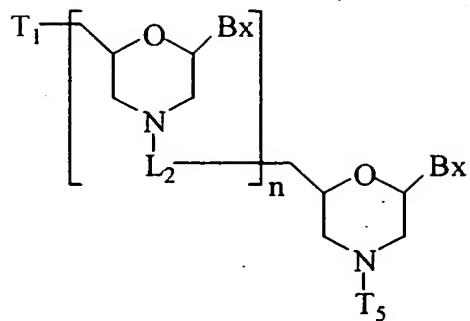
 Z₂ is hydrogen, C₁-C₆ alkyl, an amino protecting group, -C(=O)-(CH₂)_n-J-Z₃, a D or L α -amino acid linked via the α -carboxyl group or optionally through the ω -carboxyl group

when the amino acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D and L amino acids linked through a carboxyl group;

Z_3 is hydrogen, an amino protecting group, $-C_1-C_6$ alkyl, 5 $-C(=O)-CH_3$, benzyl, benzoyl, or $-(CH_2)_n-N(H)Z_1$; each J is O, S or NH; R_5 is a carbonyl protecting group; and n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been 10 studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking 15 groups have been selected to give a non-ionic oligomeric compound. The non-ionic morpholino-based oligomeric compounds are less likely to have undesired interactions with cellular proteins. Morpholino-based oligomeric compounds are non-ionic mimics of oligonucleotides which 20 are less likely to form undesired interactions with cellular proteins (Dwaine A. Braasch and David R. Corey, *Biochemistry*, 2002, 41(14), 4503-4510). Morpholino-based oligomeric compounds are disclosed in United States Patent 25 5,034,506, issued July 23, 1991. The morpholino class of oligomeric compounds have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups (L_2) joining the monomeric subunits. The basic formula is shown below:

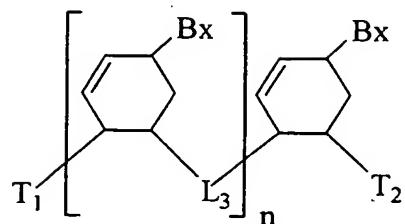


wherein

5 T_1 is hydroxyl or a protected hydroxyl;
 T_5 is hydrogen or a phosphate or phosphate derivative;
 L_2 is a linking group; and
 n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred
 10 to as cyclohexenyl nucleic acids (CeNA). The furanose ring
 normally present in an DNA/RNA molecule is replaced with a
 cyclohexenyl ring. CeNA DMT protected phosphoramidite
 monomers have been prepared and used for oligomeric
 compound synthesis following classical phosphoramidite
 15 chemistry. Fully modified CeNA oligomeric compounds and
 oligonucleotides having specific positions modified with
 CeNA have been prepared and studied (see Wang et al., *J.
 Am. Chem. Soc.*, 2000, 122, 8595-8602). In general the
 incorporation of CeNA monomers into a DNA chain increases
 20 its stability of a DNA/RNA hybrid. CeNA oligoadenylates
 formed complexes with RNA and DNA complements with similar
 stability to the native complexes. The study of
 incorporating CeNA structures into natural nucleic acid
 structures was shown by NMR and circular dichroism to
 25 proceed with easy conformational adaptation. Furthermore
 the incorporation of CeNA into a sequence targeting RNA was
 stable to serum and able to activate E. Coli RNase
 resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



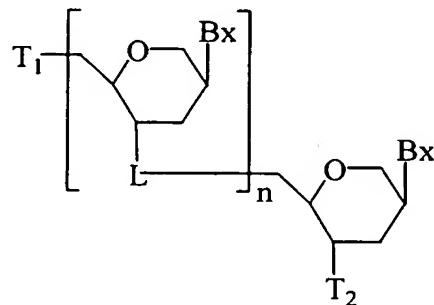
5 wherein

each Bx is a heterocyclic base moiety;

T₁ is hydroxyl or a protected hydroxyl; and

T₂ is hydroxyl or a protected hydroxyl.

Another class of oligonucleotide mimetic
 10 (anhydrohexitol nucleic acid) can be prepared from one or
 more anhydrohexitol nucleosides (see, Wouters and
 Herdewijn, *Bioorg. Med. Chem. Lett.*, 1999, 9, 1563-1566)
 and would have the general formula:

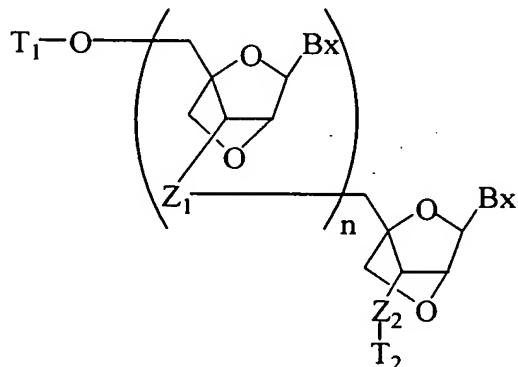


15

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a
 20 bicyclic sugar moiety. The linkage is preferably a methylene (-CH₂-)_n group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2 (Singh et al., *Chem. Commun.*, 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA

and RNA ($T_m = +3$ to $+10$ C), stability towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:

5



The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the 10 LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved 15 stacking of the nucleobases (Wengel et al., Nucleosides Nucleotides, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., J. Am. Chem. Soc., 1998, 120, 13252-13253). LNA:LNA hybridization was shown to be the 20 most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points ($T_m = +15/+11$) toward DNA complements. The universality of LNA-mediated 25 hybridization has been stressed by the formation of exceedingly stable LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational

restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) 5 spectra show that duplexes involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been 10 demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

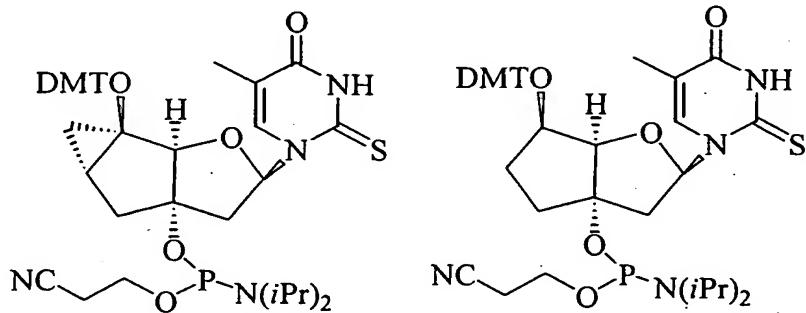
Novel types of LNA-oligomeric compounds, as well as 15 the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement oligomers, substrates for nucleic acid polymerases and generally as nucleotide based drugs. Potent and nontoxic 20 antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638.) The authors have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily in 25 blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor signaling in living rat brain and detection of reporter genes in *Escherichia coli*. Lipofectin-mediated efficient delivery of LNA into 30 living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid

recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., 10 PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In 15 addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Further oligonucleotide mimetics have been prepared to 20 include bicyclic and tricyclic nucleoside analogs having the formulas (amidite monomers shown):

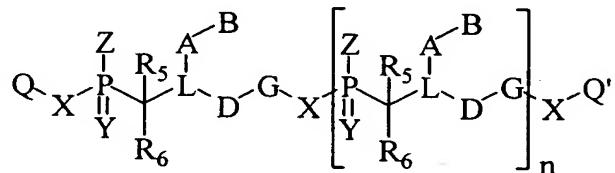


(see Steffens et al., *Helv. Chim. Acta*, 1997, 80, 2426-25 2439; Steffens et al., *J. Am. Chem. Soc.*, 1999, 121, 3249-3255; and Renneberg et al., *J. Am. Chem. Soc.*, 2002, 124, 5993-6002). These modified nucleoside analogs have been oligomerized using the phosphoramidite approach and the

resulting oligomeric compounds containing tricyclic nucleoside analogs have shown increased thermal stabilities (T_m's) when hybridized to DNA, RNA and itself. Oligomeric compounds containing bicyclic nucleoside analogs have shown 5 thermal stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus group in a backbone the backbone. This class of oligonucleotide mimetic is reported to have useful physical 10 and biological and pharmacological properties in the areas of inhibiting gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

15 The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in their entirety) is shown below.



20

Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

25 *Modified sugars*

Oligomeric compounds of the invention may also contain one or more substituted sugar moieties. Preferred oligomeric compounds comprise a sugar substituent group selected from: OH; F; O-, S-, or N-alkyl; O-, S-, or N-30 alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or

unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10.

5 Other preferred oligonucleotides comprise a sugar substituent group selected from: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂,

10 heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an

15 oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further

20 preferred modification includes 2'-dimethylaminoxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-aminoethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂.

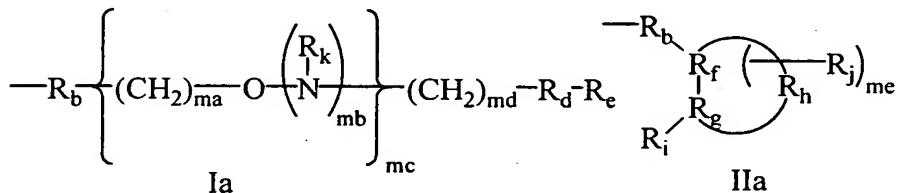
25 Other preferred sugar substituent groups include methoxy (-O-CH₃), aminopropoxy (-OCH₂CH₂CH₂NH₂), allyl (-CH₂-CH=CH₂), -O-allyl (-O-CH₂-CH=CH₂) and fluoro (F). 2'-Sugar substituent groups may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification

30 is 2'-F. Similar modifications may also be made at other positions on the oligomeric compound, particularly the 3' position of the sugar on the 3' terminal nucleoside or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligomeric compounds may also have

sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:

5 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 10 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 15 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Further representative sugar substituent groups include groups of formula Ia or IIa:



15

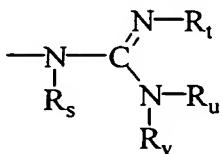
wherein:

R_b is O, S or NH;

R_d is a single bond, O, S or C(=O);

R_e is C₁-C₁₀ alkyl, N(R_k)(R_m), N(R_k)(R_n), N=C(R_p)(R_q),

20 N=C(R_p)(R_r) or has formula IIIa;



R_p and R_q are each independently hydrogen or C₁-C₁₀ alkyl;

R_r is -R_x-R_y;

25 each R_s, R_t, R_u and R_v is, independently, hydrogen, C(O)R_w, substituted or unsubstituted C₁-C₁₀ alkyl, substituted or unsubstituted C₂-C₁₀ alkenyl, substituted or

unsubstituted C₂-C₁₀ alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy,

5 halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_u and R_v, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_w is, independently, substituted or unsubstituted C₁-C₁₀ alkyl, trifluoromethyl, cyanoethoxy, methoxy, 10 ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_x is hydrogen, a nitrogen protecting group or -R_x-R_y;

R_p is hydrogen, a nitrogen protecting group or -R_x-R_y;

15 R_x is a bond or a linking moiety;

R_y is a chemical functional group, a conjugate group or a solid support medium;

each R_m and R_n is, independently, H, a nitrogen protecting group, substituted or unsubstituted C₁-C₁₀ alkyl,

20 substituted or unsubstituted C₂-C₁₀ alkenyl, substituted or unsubstituted C₂-C₁₀ alkynyl, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, alkynyl; NH₃⁺, N(R_u)(R_v), guanidino and acyl where 25 said acyl is an acid amide or an ester;

or R_m and R_n, together, are a nitrogen protecting group, are joined in a ring structure that optionally includes an additional heteroatom selected from N and O or are a chemical functional group;

30 R_i is OR_z, SR_z, or N(R_z)₂;

each R_z is, independently, H, C₁-C₈ alkyl, C₁-C₈ haloalkyl, C(=NH)N(H)R_u, C(=O)N(H)R_u or OC(=O)N(H)R_u;

R_f, R_g and R_h comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6

carbon atoms and 1 or 2 heteroatoms wherein said heteroatoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated

5 heterocyclic;

R_j is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, N(R_k)(R_m) OR_k, halo, SR_k or CN;

10 m_a is 1 to about 10;

each m_b is, independently, 0 or 1;

m_c is 0 or an integer from 1 to 10;

m_d is an integer from 1 to 10;

m_e is from 0, 1 or 2; and

15 provided that when m_c is 0, m_d is greater than 1.

Representative substituents groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by 20 reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Oligomeric compounds that are Conformationally 25 Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred sugar substituent groups include O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 30 1 to about 10.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled

"Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent 6,147,200 which is hereby 5 incorporated by reference in its entirety.

Representative dimethylaminoethoxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethoxyethyl-Oligomeric compounds", filed August 6, 1999, hereby 10 incorporated by reference in its entirety.

Modified Nucleobases/Naturally occurring nucleobases

Oligomeric compounds may also include nucleobase (often referred to in the art simply as "base" or 15 "heterocyclic base moiety") modifications or substitutions.

As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as 20 heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives 25 of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH₃) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiolo, 8-thioalkyl, 8-hydroxyl and other 8-substituted 30 adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-

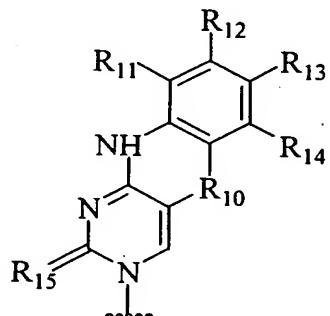
deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859,

10 Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., 15 ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and 20 are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

In one aspect of the present invention oligomeric compounds are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding

properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general 5 formula:



Representative cytosine analogs that make 3 hydrogen 10 bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one ($R_{10} = O$, $R_{11} - R_{14} = H$) [Kurchavov, et al., *Nucleosides and Nucleotides*, 1997, 16, 1837-1846], 1,3-diazaphenothiazine-2-one ($R_{10} = S$, $R_{11} - R_{14} = H$), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. *Am. Chem. Soc.* 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one ($R_{10} = O$, $R_{11} - R_{14} = F$) [Wang, J.; Lin, K.-Y., Matteucci, M. *Tetrahedron Lett.* 1998, 39, 8385-8388]. Incorporated into oligonucleotides these base 15 modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see U.S. Patent Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this 20 application and are herein incorporated by reference in

their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxyazine-2-one scaffold ($R_{10} = O$, $R_{11} = -O-(CH_2)_2-NH_2$, $R_{12-14}=H$) [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine (dC5^{me}), which is the highest known affinity enhancement for a single modification, yet. On the other hand, the gain in helical stability does not compromise the specificity of the oligonucleotides. The T_m data indicate an even greater discrimination between the perfect match and mismatched sequences compared to dC5^{me}. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact,

promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity

5 [Lin, K-Y; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.;

10 Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these

15 heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful as heterocyclic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as

20 U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and United States Patent Application Serial number 09/996,292 filed November 28, 2001, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

The oligonucleotides of the present invention also

30 include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This

may be done at any of the positions of the oligonucleotide. Thus, a 20-mer may comprise 60 variations (20 positions x 3 alternates at each position) in which the original nucleotide is substituted with any of the three alternate 5 nucleotides. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of HCV mRNA and/or HCV replication.

Conjugates

10 A further preferred substitution that can be appended to the oligomeric compounds of the invention involves the linkage of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting oligomeric compounds. In one embodiment such 15 modified oligomeric compounds are prepared by covalently attaching conjugate groups to functional groups such as hydroxyl or amino groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, 20 groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluores- 25 ceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA.

30 Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed

October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a

5 thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a

10 phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or

15 adamantan acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety

20 (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

The oligomeric compounds of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, 30 ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a

sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is 5 incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 10 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 15 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 15 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 20 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric oligomeric compounds

25 It is not necessary for all positions in an oligomeric compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligomeric compound or even at a single monomeric subunit such as a nucleoside within a oligomeric 30 compound. The present invention also includes oligomeric compounds which are chimeric oligomeric compounds. "Chimeric" oligomeric compounds or "chimeras," in the context of this invention, are oligomeric compounds that contain two or more chemically distinct regions, each made

up of at least one monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased 5 resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, 10 RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be 15 obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, 20 associated nucleic acid hybridization techniques known in the art.

Chimeric oligomeric compounds of the invention may be formed as composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides 25 and/or oligonucleotide mimetics as described above. Such oligomeric compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not 30 limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its

entirety.

3'-endo modifications

In one aspect of the present invention oligomeric compounds include nucleosides synthetically modified to induce a 3'-endo sugar conformation. A nucleoside can incorporate synthetic modifications of the heterocyclic base, the sugar moiety or both to induce a desired 3'-endo sugar conformation. These modified nucleosides are used to mimic RNA like nucleosides so that particular properties of an oligomeric compound can be enhanced while maintaining the desirable 3'-endo conformational geometry. There is an apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a requirement (e.g. trigger) of RNA interference which is supported in part by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears efficient in triggering RNAi response in the *C. elegans* system. Properties that are enhanced by using more stable 3'-endo nucleosides include but aren't limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage. The present invention provides oligomeric triggers of RNAi having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation.

Scheme 1



C2'-endo/Southern

C3'-endo/Northern

5 Nucleoside conformation is influenced by various factors including substitution at the 2', 3' or 4'-positions of the pentofuranosyl sugar. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the

10 equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984, Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2, below (Gallo et al.,

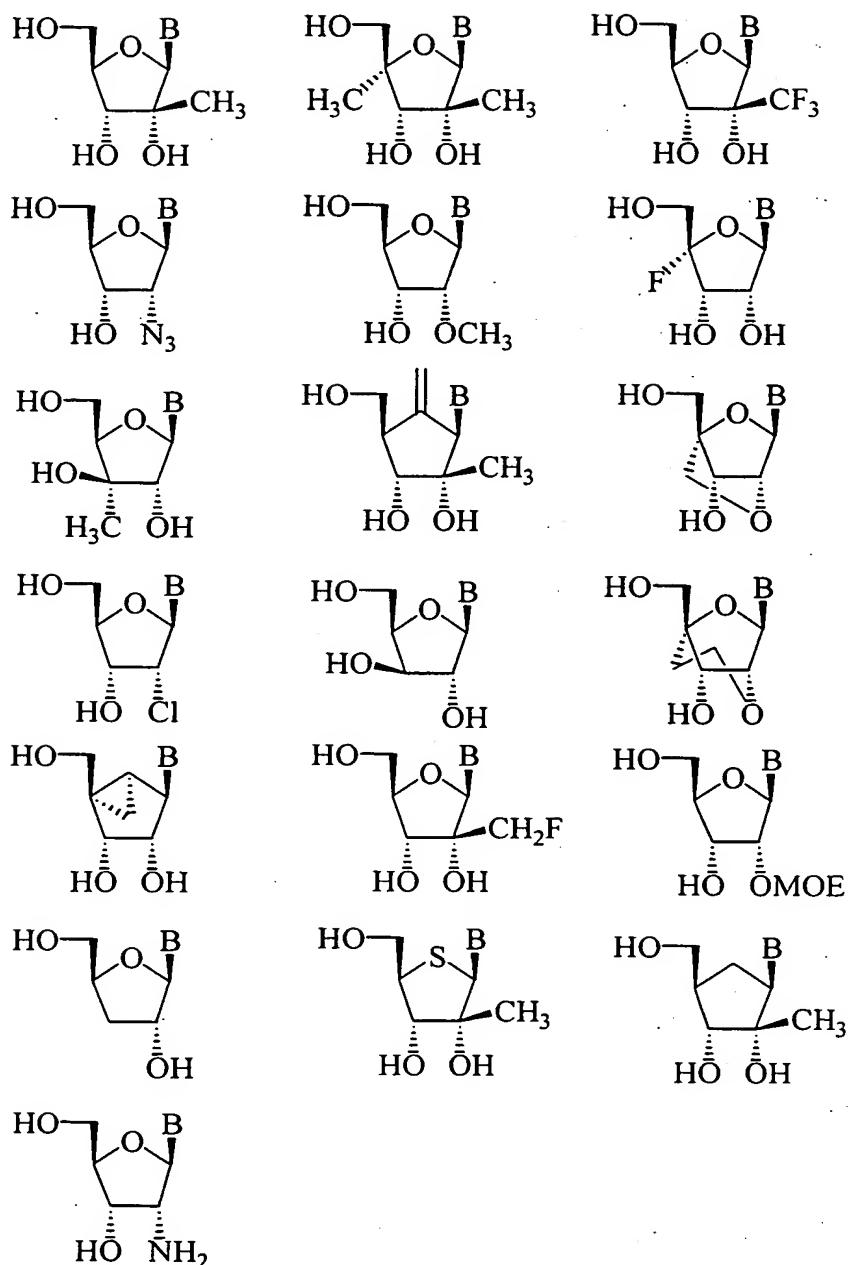
15 Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.) Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'deoxy-2'F-nucleosides

20 (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution at the 4'-position to give 4'-F modified nucleosides

25 (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarba nucleoside analogs (Jacobson et al., J. Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al.,

Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides 5 modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73- 10 76.) Examples of modified nucleosides amenable to the present invention are shown below in Table I. These examples are meant to be representative and not exhaustive.

Table I



5

The preferred conformation of modified nucleosides and their oligomers can be estimated by various methods such as molecular dynamics calculations, nuclear magnetic resonance spectroscopy and CD measurements. Hence, modifications

predicted to induce RNA like conformations, A-form duplex geometry in an oligomeric context, are selected for use in the modified oligonucleotides of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known in the art (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press., and the examples section below.) Nucleosides known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B

10 In one aspect, the present invention is directed to oligonucleotides that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligonucleotide is 15 selected having an effective length and sequence that is complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides 20 with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much cheaper and easier to synthesize and/or incorporate into an oligonucleotide. The selected sequence 25 can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the 5' and 3'-termini as there are often advantageous modifications that can be made to one or more 30 of the terminal nucleosides. The oligomeric compounds of the present invention include at least one 5'-modified phosphate group on a single strand or on at least one 5'-position of a double stranded sequence or sequences. Further modifications are also considered such as

internucleoside linkages, conjugate groups, substitute sugars or bases, substitution of one or more nucleosides with nucleoside mimetics and any other modification that can enhance the selected sequence for its intended target.

5 The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and "B Form" for DNA. The respective conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins,

10 *Biochem. Biophys. Res. Comm.*, 1970, 47, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (Tm's) than DNA:DNA duplexes (Sanger et al., *Principles of Nucleic Acid Structure*, 1984, Springer-Verlag; New York, NY.; Lesnik et al., *Biochemistry*, 1995, 15, 34, 10807-10815; Conte et al., *Nucleic Acids Res.*, 1997, 25, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., *Nucleic Acids Res.*, 1993, 21, 20 2051-2056). The presence of the 2' hydroxyl in RNA biases the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2' hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that

25 help stabilize the RNA duplex (Egli et al., *Biochemistry*, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, et. al., *Nucleic Acids Research*, 1998, 26, 2473-30 2480, who pointed out that in considering the furanose

conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less 5 stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al.*, *Nucleic Acids Res.*, 1993, 21, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may 10 result in poor stacking interactions (Lane *et al.*, *Eur. J. Biochem.*, 1993, 215, 297-306; Fedoroff *et al.*, *J. Mol. Biol.*, 1993, 233, 509-523; Gonzalez *et al.*, *Biochemistry*, 1995, 34, 4969-4982; Horton *et al.*, *J. Mol. Biol.*, 1996, 264, 521-533). The stability of the duplex formed between 15 a target RNA and a synthetic sequence is central to therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA 20 requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

25 One routinely used method of modifying the sugar puckering is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A 30 number of different substituents have been studied to determine their sugar puckering effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest

population (7%). The populations of adenosine (2'-OH) versus deoxyadenosine (2'-H) are 36% and 19%, respectively.

Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine -

5 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation.

As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed

10 that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form duplex. Data from UV hypochromicity, circular dichroism, and ¹H NMR also indicate that the degree of stacking decreases as the

15 electronegativity of the halo substituent decreases.

Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to exert a number

20 of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities.

These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding.

30 However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-OCH₂CH₂OCH₃)

side chain (Baker *et al.*, *J. Biol. Chem.*, 1997, 272, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as O-methyl, O-propyl, and O-aminopropyl. Oligonucleotides having the 2'-O-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P., *Helv. Chim. Acta*, 1995, 78, 486-504; Altmann *et al.*, *Chimia*, 1996, 50, 10 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, 1996, 24, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, 16, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE modification displayed improved RNA affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

25 **Chemistries Defined**

Unless otherwise defined herein, alkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where possible) branched chain aliphatic hydrocarbyl.

Unless otherwise defined herein, heteroalkyl means C₁-C₁₂, preferably C₁-C₈, and more preferably C₁-C₆, straight or (where possible) branched chain aliphatic hydrocarbyl containing at least one, and preferably about 1 to about 3, hetero atoms in the chain, including the terminal portion of the chain.. Preferred heteroatoms include N, O and S.

Unless otherwise defined herein, cycloalkyl means C₃-C₁₂, preferably C₃-C₈, and more preferably C₃-C₆, aliphatic hydrocarbyl ring.

Unless otherwise defined herein, alkenyl means C₂-C₁₂, 5 preferably C₂-C₈, and more preferably C₂-C₆ alkenyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon double bond.

Unless otherwise defined herein, alkynyl means C₂-C₁₂, 10 preferably C₂-C₈, and more preferably C₂-C₆ alkynyl, which may be straight or (where possible) branched hydrocarbyl moiety, which contains at least one carbon-carbon triple bond.

Unless otherwise defined herein, heterocycloalkyl 15 means a ring moiety containing at least three ring members, at least one of which is carbon, and of which 1, 2 or three ring members are other than carbon. Preferably the number of carbon atoms varies from 1 to about 12, preferably 1 to about 6, and the total number of ring members varies from 20 three to about 15, preferably from about 3 to about 8.

Preferred ring heteroatoms are N, O and S. Preferred heterocycloalkyl groups include morpholino, thiomorpholino, piperidinyl, piperazinyl, homopiperidinyl, homopiperazinyl, homomorpholino, homothiomorpholino, pyrrolodinyl, 25 tetrahydrooxazolyl, tetrahydroimidazolyl, tetrahydrothiazolyl, tetrahydroisoxazolyl, tetrahydropyrazolyl, furanyl, pyranyl, and tetrahydroisothiazolyl.

Unless otherwise defined herein, aryl means any 30 hydrocarbon ring structure containing at least one aryl ring. Preferred aryl rings have about 6 to about 20 ring carbons. Especially preferred aryl rings include phenyl, napthyl, anthracenyl, and phenanthrenyl.

Unless otherwise defined herein, hetaryl means a ring

moiety containing at least one fully unsaturated ring, the ring consisting of carbon and non-carbon atoms. Preferably the ring system contains about 1 to about 4 rings.

Preferably the number of carbon atoms varies from 1 to 5 about 12, preferably 1 to about 6, and the total number of ring members varies from three to about 15, preferably from about 3 to about 8. Preferred ring heteroatoms are N, O and S. Preferred hetaryl moieties include pyrazolyl, thiophenyl, pyridyl, imidazolyl, tetrazolyl, pyridyl, 10 pyrimidinyl, purinyl, quinazolinyl, quinoxalinyl, benzimidazolyl, benzothiophenyl, etc.

Unless otherwise defined herein, where a moiety is defined as a compound moiety, such as hetarylalkyl (hetaryl and alkyl), aralkyl (aryl and alkyl), etc., each of the 15 sub-moieties is as defined herein.

Unless otherwise defined herein, an electron withdrawing group is a group, such as the cyano or isocyanato group that draws electronic charge away from the carbon to which it is attached. Other electron withdrawing 20 groups of note include those whose electronegativities exceed that of carbon, for example halogen, nitro, or phenyl substituted in the ortho- or para-position with one or more cyano, isothiocyanato, nitro or halo groups.

Unless otherwise defined herein, the terms halogen and 25 halo have their ordinary meanings. Preferred halo (halogen) substituents are Cl, Br, and I.

The aforementioned optional substituents are, unless otherwise herein defined, suitable substituents depending upon desired properties. Included are halogens (Cl, Br, 30 I), alkyl, alkenyl, and alkynyl moieties, NO₂, NH₃ (substituted and unsubstituted), acid moieties (e.g. -CO₂H, -OSO₃H₂, etc.), heterocycloalkyl moieties, hetaryl moieties, aryl moieties, etc. In all the preceding formulae, the squiggle (~) indicates a bond to an oxygen or sulfur of the

5'-phosphate.

Phosphate protecting groups include those described in US Patents No. US 5,760,209, US 5,614,621, US 6,051,699, US 6,020,475, US 6,326,478, US 6,169,177, US 6,121,437, US 5 6,465,628 each of which is expressly incorporated herein by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for 10 such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the 15 phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

20 The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United 25 States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Patents 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 30 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated

by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are

prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid,

naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. 5 Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or 10 hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and 15 spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic 20 acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, 25 polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, 30 preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of Survivin is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective

amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, 5 inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding Survivin, enabling sandwich and other assays to easily be constructed to exploit this fact.

10 Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding Survivin can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable 15 detection means. Kits using such detection means for detecting the level of Survivin in a sample may also be prepared.

20 The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes 25 including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal, intradermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, 30 subcutaneous, intraperitoneal or intramuscular injection, drip or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, 5 powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water 10 or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Compositions for oral administration also include pulsatile delivery compositions and bioadhesive composition as described in 15 copending U.S. Patent Application Serial Nos. 09/944,493, filed August 22, 2001, and 09/935,316, filed August 22, 2001, the entire disclosures of which are incorporated herein by reference.

Compositions and formulations for parenteral, 20 intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

25 Pharmaceutical compositions and/or formulations comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1, 1-33). One or more penetration enhancers

from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, 5 linoleic acid, linolenic acid, dicaprate, tricaprate, recinoleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically 10 acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1, 1-33; El-Hariri et al., *J. Pharm. 15 Pharmacol.*, 1992, 44, 651-654). Examples of some presently preferred fatty acids are sodium caprate and sodium laurate, used singly or in combination at concentrations of 0.5 to 5%.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble 20 vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" 25 includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Examples of presently preferred bile salts are chenodeoxycholic acid (CDCA) and/or ursodeoxycholic acid (UDCA), generally used at concentrations of 0.5 to 2%.

30 Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and 5 N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51). Chelating agents have the added 10 advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-191); and perfluorochemical 15 emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-191); and non-steroidal anti-inflammatories such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic 25 acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic 30 acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid

recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated 5 oligonucleotide in hepatic tissue is reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 10 1996, 6, 177-183).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more 15 nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given 20 pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinyl-pyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, 25 pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium 30 benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents

4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, 1995, 6, 698-708).

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to

daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, 5 hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 10 5-azacytidine, hydroxyurea, deoxycouformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUDR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, 15 cisplatin, carboplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). 20 25 Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially. 30

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Examples of antisense oligonucleotides include, but are not limited to, those directed to the following targets as disclosed in the indicated U.S. Patents, or pending U.S. applications, which are commonly owned with the instant application and are hereby incorporated by reference, or the indicated published PCT applications: raf (WO 96/39415, WO 95/32987 and U.S. Patents 5,563,255 and 5,656,612), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent 5,656,743), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO 95/10938 and U.S. Patent 5,510,239), subunits of transcription factor AP-1 (pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (pending application U.S. Serial No. 08/910,629, filed August 13, 1997), MDR-1 (multidrug resistance glycoprotein; pending application U.S. Serial No. 08/731,199, filed September 30, 1997), HIV (U.S. Patents 5,166,195 and 5,591,600), herpesvirus (U.S. Patents 5,248,670 and 5,514,577), cytomegalovirus (U.S. Patents 5,442,049 and 5,591,720), papillomavirus (U.S. Patent 5,457,189), intercellular adhesion molecule-1 (ICAM-1) (U.S. Patent 5,514,788), 5-lipoxygenase (U.S. Patent 5,530,114) and influenza virus (U.S. Patent 5,580,767). Two or more combined compounds may be used together or sequentially.

In a preferred embodiment, the compounds of the present invention are used to treat a hyperproliferative condition. In a particularly preferred embodiment, the hyperproliferative condition is cancer. Types of cancers include, but are not limited to, colorectal, melanoma, liposarcoma, mesothelioma, sarcoma, pancreatic, lung, bladder, breast and prostate.

The formulation of therapeutic compositions and their

subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several 5 months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition 10 rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more 15 daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be 20 desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μ g to 100 g per kg of body weight, once or more daily, to once every 20 years.

25 While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

30 **EXAMPLES**

Example 1

Nucleoside phosphoramidites for oligonucleotide synthesis

deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham, MA or Glen Research, Inc. Sterling, VA).
5 Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of
10 tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen
15 Research, Sterling VA or ChemGenes, Needham, MA).

2'-Fluoro amidites**2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described
20 previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and U. S. Patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as
25 starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP)
30 intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and 5 conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine.

10 Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

15 Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

20

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard 25 procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are 30 prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated 5 to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether 10 was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60EC at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 15 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white 20 solid, mp 222-4EC).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 25 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160EC. After heating for 48 hours at 155-160EC, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble 30 salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/Acetone/MeOH (20:5:3) containing 0.5% Et₃NH.

The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in CHCl_3 (1.5 L) and extracted with 2x500 mL of saturated NaHCO_3 and 2x500 mL of saturated NaCl . The organic phase was dried over Na_2SO_4 , filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with $\text{EtOAc}/\text{Hexane}/\text{Acetone}$ (5:5:1) containing 0.5% Et_3NH . The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by

tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35EC. The residue was dissolved in CHCl₃ (800 mL) and extracted with 5 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel 10 column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

15 **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside.

20 Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5EC and stirred for 0.5 hours using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10EC, and the resulting mixture stirred for an additional 2 hours. The first solution was 25 added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by 30 filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

15

2'-(Aminooxyethyl) nucleoside amidites and 2'-(dimethylaminoxyethyl) nucleoside amidites

Aminooxyethyl and dimethylaminoxyethyl amidites are prepared as per the methods of United States patent applications serial number 10/037,143, filed February 14, 1998, and serial number 09/016,520, filed January 30, 1998, each of which is commonly owned with the instant application and is herein incorporated by reference.

25 **Example 2**

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of

the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55EC (18 hours), the oligonucleotides were 5 purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as 10 described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

15 Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

20 Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference. 3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

25 Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

30 Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside synthesis

Methylenemethylimino linked oligonucleosides, also

identified as MMI linked oligonucleosides, methylenedimethyl-hydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 10 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

15 Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

20 PNA synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. 25 They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

30 Synthesis of chimeric oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between

5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art 5 as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

**[2'-O-Me]-[2'-deoxy]-[2'-O-Me]chimeric
10 phosphorothioate oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are 15 synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and 20 base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hours at 25 room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hours at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by 30 rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]-[2'-deoxy]-[2'-O-(Methoxyethyl)] **chimeric** **phosphorothioate**
oligonucleotides

5 [2'-O-(2-methoxyethyl)]-[2'-deoxy]-[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

10 **[2'-O-(2-Methoxyethyl) Phosphodiester]-[2'-deoxy Phosphorothioate]-[2'-O-(2-Methoxyethyl) Phosphodiester] chimeric oligonucleotides**

15 [2'-O-(2-methoxyethyl) phosphodiester]-[2'-deoxy phosphorothioate]-[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4,1,2 20 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

25 Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to U. S. Patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide isolation

30 After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl. with 2.5 volumes ethanol. Synthesized

oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in 5 synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC 10 purified material.

Example 7**Oligonucleotide synthesis - 96 well plate format**

Oligonucleotides were synthesized via solid phase P(III) 15 phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization 20 utilizing 3, H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster 25 City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature 30 (55-60EC) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8**Oligonucleotide analysis - 96 well plate format**

The concentration of oligonucleotide in each well was
5 assessed by dilution of samples and UV absorption
spectroscopy. The full-length integrity of the individual
products was evaluated by capillary electrophoresis (CE) in
either the 96 well format (Beckman P/ACEJ MDQ) or, for
10 individually prepared samples, on a commercial CE apparatus
(e.g., Beckman P/ACEJ 5000, ABI 270). Base and backbone
composition was confirmed by mass analysis of the compounds
utilizing electrospray-mass spectroscopy. All assay test
plates were diluted from the master plate using single and
15 multi-channel robotic pipettors. Plates were judged to be
acceptable if at least 85% of the compounds on the plate were
at least 85% full length.

Example 9**Cell culture and oligonucleotide treatment**

20 The effect of antisense compounds on target nucleic acid
expression can be tested in any of a variety of cell types
provided that the target nucleic acid is present at measurable
levels. This can be routinely determined using, for example,
PCR or Northern blot analysis. The following four cell types
25 are provided for illustrative purposes, but other cell types
can be routinely used.

T-24 cells:

30 The transitional cell bladder carcinoma cell line T-24
was obtained from the American Type Culture Collection (ATCC)
(Manassas, VA). T-24 cells were routinely cultured in
complete McCoy's 5A basal media (Gibco/Life Technologies,
Gaithersburg, MD) supplemented with 10% fetal calf serum
(Gibco/Life Technologies, Gaithersburg, MD), penicillin 100

units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates 5 (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and 10 oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, 15 VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). 20 Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained 25 from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

30

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics

Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

5 3T3-L1 cells:

The mouse embryonic adipocyte-like cell line 3T3-L1 was obtained from the American Type Culture Collection (Manassas, VA). 3T3-L1 cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) 10 supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 80% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 4000 cells/well for use in RT-15 PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

20

Treatment with antisense compounds:

When cells reached 80% confluence, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEMJ-1 reduced-serum 25 medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEMJ-1 containing 3.75 μ g/mL LIPOFECTINJ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide 30 treatment. The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control

oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control 5 oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCAAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of H- 10 ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration 15 of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

20

Example 10

Analysis of oligonucleotide inhibition of Survivin expression

Antisense modulation of Survivin expression can be assayed in a variety of ways known in the art. For example, 25 Survivin mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are 30 taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*,

Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM® 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster 5 City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

Survivin protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-10 activated cell sorting (FACS). Antibodies directed to Survivin can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for 15 preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular 20 Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-25 10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) 30 are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11**Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70EC was added to each well, the plate was incubated on a 90EC hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12**Total RNA isolation**

Total mRNA was isolated using an RNEASY 96J kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the

cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three 5 times up and down. The samples were then transferred to the RNEASY 96J well plate attached to a QIAVACJ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96J plate and the vacuum 10 again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96J plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVACJ manifold 15 and blotted dry on paper towels. The plate was then re-attached to the QIAVACJ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step 20 was repeated with an additional 60 μ L water.

Example 13

Real-time quantitative PCR analysis of Survivin mRNA levels

Quantitation of Survivin mRNA levels was determined by 25 real-time quantitative PCR using the ABI PRISMJ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain 30 reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by

including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISMJ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 μ L PCR cocktail (1x TAQMAM buffer A, 5.5 mM MgCl₂, 300 μ M each of dATP, dCTP and dGTP, 600 μ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLDJ, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μ L poly(A) mRNA solution. The RT reaction was carried out by

incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). Probes and primers to human Survivin were designed to hybridize to a human Survivin sequence, using published sequence information (GenBank accession number U75285, incorporated herein as SEQ ID NO:3). For human Survivin the PCR primers were:

10 forward primer: AAGGACCACCGCATCTCTACA (SEQ ID NO: 4)
reverse primer: CCAAGTCTGGCTCGTTCTCAGT (SEQ ID NO: 5) and the PCR probe was: FAM-CGAGGCTGGCTTCATCCACTGCC-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CAAGCTTCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Probes and primers to mouse Survivin were designed to hybridize to a mouse Survivin sequence, using published sequence information (GenBank accession number AB013819, incorporated herein as SEQ ID NO: 10). For mouse Survivin the PCR primers were:

forward primer: CCGAGAACGAGCCTGATTTG (SEQ ID NO: 11)
reverse primer: GGGAGTGCTTCTATGCTCCTCTA (SEQ ID NO: 12) and the PCR probe was: FAM-TAAGGAATTGGAAGGCTGGGAACCCG-TAMRA (SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and TAMRA, (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were:

forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14) reverse primer: GGGTCTCGCTCCTGGAAAGCT (SEQ ID NO: 15) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTAC-TAMRA 3' (SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of Survivin mRNA levels

10 Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOLJ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by 15 electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBONDJ-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern 20 Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKERJ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then 25 probed using QUICKHYBJ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human Survivin, a human Survivin specific probe was prepared by PCR using the forward primer AAGGACCACCGCATCTCTACA (SEQ ID NO: 4) and the reverse primer 30 CCAAGTCTGGCTCGTTCTCAGT (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse Survivin, a mouse Survivin specific

probe was prepared by PCR using the forward primer CCGAGAACGAGCCTGATTG (SEQ ID NO: 11) and the reverse primer GGGAGTGCTTCTATGCTCCTCTA (SEQ ID NO: 12). To normalize for variations in loading and transfer efficiency membranes were 5 stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Example 15

Antisense inhibition of Survivin expression- phosphorothioate 10 oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Survivin RNA, using published sequences (GenBank accession number U75285, incorporated herein as SEQ ID NO: 3). 15 The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U75285), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones 20 (internucleoside linkages) throughout. All cytidines are 5-methylcytidines. The compounds were analyzed for effect on Survivin mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

25

TABLE 1
Inhibition of human Survivin mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
23625	5' UTR	1	gcgattcaaattctggcgg	0	17
23653	5' UTR	19	cctctgccaacgggtccc	4	18
23654	5' UTR	75	tgagaaagggtgcagg	46	19

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
23655	5' UTR	103	ttcttgaatgttagagatg	0	20
23656	5' UTR	128	ggcgcagccctccaagaa	38	21
23657	Coding	194	caagtctggctcgttctc	0	22
23658	Coding	226	tccagtccttgaagcag	32	23
23659	Coding	249	ggtcgtcatctggctccc	36	24
23660	Coding	306	gcttcttgacagaaaagga	35	25
23661	Coding	323	ggtaattcttcaaactg	0	26
23662	Coding	363	tcttgctcttctctgt	34	27
23663	Coding	393	tcttattgttggttcct	0	28
23664	Coding	417	tcgcagttcctcaaatt	37	29
23665	Coding	438	cgtggcacggcgcactt	72	30
23666	Coding	511	cctggaagtggcagcc	16	31
23667	Coding	542	acaggaaggctggcggca	70	32
23668	Coding	587	tttggaaaatgttcatctc	8	33
23669	Coding	604	acagtgtaaaacatcta	0	34
23670	Coding	625	ctttcaagacaaaacagg	0	35
23671	Coding	650	acaggcagaagcacctct	0	36
23672	Coding	682	aaggcagccactgttacca	64	37
23673	Coding	700	aaagagagagagagagag	18	38
23674	Coding	758	tccctcaactctcacctg	29	39
23675	3' UTR	777	aggcacactgccttcttc	43	40
23676	3' UTR	808	ccacgcgaacaaagctgt	62	41
23677	3' UTR	825	actgtggaggctctgcc	0	42
23678	3' UTR	867	aggactgtgacagcctca	62	43
23679	3' UTR	901	tcagattcaacaggcacc	0	44
23680	3' UTR	1016	attctctcatcacacaca	26	45
23681	3' UTR	1054	tgttgttacacagttagag	0	46
23682	3' UTR	1099	tgtgctattctgtgaatt	20	47
23683	3' UTR	1137	gacttagaatggctttgt	37	48

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
23684	3' UTR	1178	ctgtctcctcatccacct	41	49
23685	3' UTR	1216	aaaaggagtatctgccag	39	50
23686	3' UTR	1276	gaggagcggccagcatgt	47	51
23687	3' UTR	1373	ggctgacagacacacggc	41	52
23688	3' UTR	1405	ccgtgtggagaacgtgac	22	53
23689	3' UTR	1479	tacgccagacttcagccc	1	54
23690	3' UTR	1514	atgacagggaggaggggcg	0	55
23691	3' UTR	1571	gccgagatgacctccaga	66	56

As shown in Table 1, SEQ ID NOs: 19, 21, 23, 24, 25, 27, 29, 30, 32, 37, 40, 41, 43, 48, 49, 50, 51, 52 and 56 demonstrated at least 30% inhibition of Survivin expression in this assay and are therefore preferred.

Example 16

Antisense inhibition of Survivin expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human Survivin were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (GenBank accession no. U75285), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

5

TABLE 2
Inhibition of human Survivin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET	SEQUENCE	%	SEQ ID
					Inhibition NO
23692	5' UTR	1	gcgattcaaattctggcgg	22	57
23693	5' UTR	19	cctctgccaacgggtccc	15	58
23694	5' UTR	75	tgagaaaagggtggccagg	11	59
23695	5' UTR	103	ttcttgaatgttagatg	37	60
23696	5' UTR	128	ggcgccagccctccaagaa	16	61
23697	Coding	194	caagtctggctcggttc	17	62
23698	Coding	226	tccagctccttgaagcag	0	63
23699	Coding	249	ggtcgtcatctggctccc	19	64
23700	Coding	306	gcttcttgacagaaaagga	35	65
23701	Coding	323	ggtaattcttcaaactg	15	66
23702	Coding	363	tcttggctctttcttgt	8	67
23703	Coding	393	tcttattgttggtttcct	41	68
23704	Coding	417	tcgcagttcctcaaatt	24	69
23705	Coding	438	cgttggcacggcgcactt	72	70
23706	Coding	511	ccttggaaagtgggtgcagcc	4	71
23707	Coding	542	acagggaaaggctggggca	48	72
23708	Coding	587	tttggaaaatgttgatctc	2	73
23709	Coding	604	acagttgaaacatctaatt	28	74
23710	Coding	625	ctttcaagacaaaacagg	0	75
23711	Coding	650	acaggcagaaggcacctct	38	76
23712	Coding	682	aaggcagccactgttacca	27	77
23713	Coding	700	aaagagagagagagagag	0	78
23714	Coding	758	tccctcacttctcacctg	0	79
23715	3' UTR	777	aggcacactgccttcttc	44	80
23716	3' UTR	808	ccacgcgaacaaagctgt	25	81
23717	3' UTR	825	actgttggaaaggctgttgc	8	82
23718	3' UTR	867	aggactgtgacagcctca	49	83
23719	3' UTR	901	tcagattcaacaggcacc	0	84
23720	3' UTR	1016	attctctcatcacacacaca	0	85
23721	3' UTR	1054	tgttgtaaacagtagag	0	86

ISIS#	REGION	TARGET	SEQUENCE	%	SEQ ID
					Inhibition NO
23722	3' UTR	1099	tgtgctattctgtgaatt	80	87
23723	3' UTR	1137	gacttagaatggcttgt	44	88
23724	3' UTR	1178	ctgtctcctcatccacct	27	89
23725	3' UTR	1216	aaaaggagtatctgccag	21	90
23726	3' UTR	1276	gaggagcggccagcatgt	39	91
23727	3' UTR	1373	ggctgacagacacacacggc	45	92
23728	3' UTR	1405	ccgtgtggagaacgtgac	24	93
23729	3' UTR	1479	tacgccagacttcagccc	25	94
23730	3' UTR	1514	atgacagggaggaggcgc	0	95
23731	3' UTR	1571	gccgagatgacacctccaga	19	96

As shown in Table 2, SEQ ID NOs: 60, 65, 68, 70, 72, 76, 80, 83, 87, 88, 91 and 92 demonstrated at least 30% inhibition of Survivin expression in this experiment and are preferred.

5

Example 17

Antisense inhibition of Survivin expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a third series 10 of oligonucleotides targeted to human Survivin mRNA were synthesized. The oligonucleotide sequences are shown in Table 3. Target sites are indicated by nucleotide numbers to which the oligonucleotide binds. The human Survivin mRNA was generated by splicing nucleotides 2811-2921, 3174-3283, 5158- 15 5275 and 11955-12044 from GenBank accession no. U75285 creating the complete human mRNA sequence herein incorporated as SEQ ID NO: 97.

All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central 20 "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All

cytidine residues are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

5

TABLE 3

Inhibition of human Survivin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
107289	Coding	14	gccaacgggtcccgcat	5	98
107290	Coding	35	catgccgcgcgcacc	4	99
107291	Coding	90	agatgcgtggtccttga	94	100
107292	Coding	110	gggcacgttcttgaatgt	14	101
107293	Coding	166	tggatgaagccagcctcg	0	102
107294	Coding	212	gcagaagaaacactgggc	0	103
107295	Coding	233	ccagccttccagctcctt	0	104
107296	Coding	283	caaccggacgaatgcttt	0	105
107297	Coding	299	gacagaaaggaaagcgca	83	106
107298	Coding	313	tcaaactgcttcttgcaca	73	107
107299	Coding	329	accaagggttaattcttc	0	108
107300	Coding	359	ggctcttctctgtccag	7	109
107301	Coding	370	attttgttcttggcttt	4	110
107302	Coding	398	tttcttcttattgttgg	11	111
107303	Coding	412	gtttcctcaaattcttc	0	112
107304	Coding	421	ttcttcgcagttcctca	49	113
107305	Coding	432	cacggcgcactttctcg	22	114
107306	Coding	445	agctgctcgatggcacgg	7	115
107307	Coding	495	ccactctgggaccaggca	0	116
107308	Coding	514	aaccctggaagtggcga	0	117
107309	Coding	529	tggcaccaggaaataaac	0	118
107310	Coding	566	tcctaagacattgctaag	1	119
107311	Coding	579	tgttgatctccttccta	3	120
107312	Coding	590	taatttggaaaatgttcat	15	121
107313	Coding	599	tgaaacatctaatttggaa	0	122
107314	Coding	613	aacaggagcacagttgaa	27	123
107315	Coding	619	agacaaaacaggagcaca	0	124
107316	Coding	630	tgccactttcaagacaaa	24	125
107317	Coding	635	tctggtgccactttcaag	0	126

ISIS #	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
107318	Coding	653	Tgcacaggcagaaggcacc	15	127
107319	Coding	676	ccactgttaccagcagca	4	128
107320	Coding	701	aaaagagagagagagaga	0	129
107321	Coding	766	cttcttcctccctcactt	7	130
107322	Coding	789	agctctagcaaaaggac	0	131
107323	Coding	814	ctctgcccacgcgaacaa	13	132
107324	Coding	836	cagacacattcactgtgg	0	133
107325	Coding	852	tcaacaacatgaggtcca	0	134
107326	Coding	882	gccaaagtccacactcagg	0	135
107327	Coding	1039	gaggagccaggactctg	16	136
107328	Coding	1067	aataagaaagccatgttg	0	137
107329	Coding	1080	acaattcaaacaaaataa	30	138
107330	Coding	1081	aacaattcaaacaaaata	0	139
107331	Coding	1082	taacaattcaaacaaaat	3	140
107332	Coding	1083	ttaacaattcaaacaaaa	31	141
107333	Coding	1084	attaacaattcaaacaaa	9	142
107334	Coding	1085	aattaacaattcaaacaa	10	143
107335	Coding	1092	ttctgtgaattaacaatt	16	144
107336	Coding	1093	attctgtgaattaacaat	0	145
107337	Coding	1094	tattctgtgaattaacaa	25	146
107338	Coding	1095	ctattctgtgaattaaca	12	147
107339	Coding	1096	gctattctgtgaattaac	14	148
107340	Coding	1097	tgctattctgtgaattaa	14	149
107341	Coding	1098	gtgctattctgtgaatta	8	150
107342	Coding	1100	ttgtgctattctgtgaat	18	151
107343	Coding	1101	tttggctattctgtgaa	33	152
107344	Coding	1102	gtttgtgctattctgtga	11	153
107345	Coding	1103	agtttggctattctgtg	21	154
107346	Coding	1104	tagtttggctattctgt	17	155
107347	Coding	1105	gtagtttggctattctg	57	156
107348	Coding	1106	tgttagtttggctattct	6	157
107349	Coding	1107	ttttagtttggctatttc	13	158
107350	Coding	1108	attgttagtttggctatt	15	159
107351	Coding	1109	aattgttagtttggctat	0	160
107352	Coding	1110	taattgttagtttggctat	25	161
107353	Coding	1120	tgcttagtttaattgta	0	162
107354	Coding	1144	ccccaaatgacttagaaatg	7	163
107355	Coding	1163	cctgaagttcaccccggt	19	164
107356	Coding	1184	tctattctgtctcctcat	0	165
107357	Coding	1199	gacgcttcctatcactct	18	166

ISIS #	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO
107358	Coding	1222	agtggcaaaaggagtatc	0	167
107359	Coding	1239	ctgtctaatcacacagca	0	168
107360	Coding	1281	tgagggaggagcggccag	0	169
107361	Coding	1350	gcagcccagccagtcccc	0	170
107362	Coding	1379	aggttgggctgacagaca	1	171
107363	Coding	1399	ggagaacgtgacagatgt	23	172
107364	Coding	1425	gggcggactgcgtcttc	0	173
107365	Coding	1470	cttcagccctgcgggagc	0	174
107366	Coding	1488	ccatcatttacgcccaga	0	175
107367	Coding	1509	agggaggaggcgaatca	0	176
107368	Coding	1585	atttctcaggaacagccg	7	177

As shown in Table 3, SEQ ID NOs: 101, 106, 107, 113, 138, 141, 152 and 156 demonstrated at least 30% inhibition of human Survivin expression in this assay and are preferred.

5

Example 18

Antisense inhibition of mouse Survivin expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the mouse Survivin RNA, using published sequences (GenBank accession number AB013819, incorporated herein as SEQ ID NO: 10). The oligonucleotides are shown in Table 4. A target site@ indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 4 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages

are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse Survivin mRNA levels by quantitative real-time PCR as described in other examples 5 herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

TABLE 4
Inhibition of mouse Survivin mRNA levels by chimeric
10 phosphorothioate oligonucleotides having 2'-MOE wings and a
deoxy gap

ISIS #	REGION	TARGET SITE	SEQUENCE	% INHIBITION	SEQ ID NO
114968	5'UTR	3	agagccccggccccctcg	0	178
114967	5'UTR	4	gagagccccggccccctcg	0	179
114966	5'UTR	16	agagcatgccggagagccc	0	108
114965	5'UTR	25	gcgcgcgcagagcatgccc	0	181
114964	5'UTR	55	aaacgcaggattcaaatcg	0	182
114963	5'UTR	66	caagacgactcaaacgcagg	0	183
114962	5'UTR	68	gc当地agacgactcaaacgc当地	0	184
114961	Start Codon	92	catgatggcgtaaccacaac	0	185
114972	Start Codon	101	cggagctccatgatggcgt	27	186
114960	Start Codon	104	c当地ccggagctccatgatgg	47	187
114959	Coding	171	ggaagggccagttcttgaag	35	188
114958	Coding	184	gc当地cgtccctccaggaagg	0	189
114957	Coding	186	aggcgcagtc当地ccaggaag	10	190
114957	Coding	186	aggcgcagtc当地ccaggaag	6	191
114971	Coding	189	tgc当地ggcgcagtc当地ccagg	30	192
114956	Coding	249	aatcaggctcggtctcggt	46	193
114955	Coding	259	cactggccaaatcaggctc	14	194
114954	Coding	289	cagccttccaattcctaaa	0	195
114953	Coding	300	catcgggttcccagccttcc	67	196
114952	Coding	303	tgtcatcggttcccagcct	83	197
114951	Coding	315	cctctatcggttgc当地atcg	40	198
114950	Coding	327	gcttctatgctc当地tatac	39	199
114949	Coding	358	ttgacagtgaggaaggc当地a	0	200
114948	Coding	374	ttcttccatctgcttcttga	0	201
114947	Coding	387	cactgacggttagtcttcc	39	202

ISIS #	REGION	TARGET SITE	SEQUENCE	% INHIBITION	SEQ ID NO
114946	Coding	389	ttcaactgacggtagttctt	12	203
114945	Coding	394	aagaattcaactgacggtag	26	204
114944	Coding	396	tcaagaattcaactgacggtt	38	205
114943	Coding	465	cttcaaactctttgcttg	10	206
114942	Coding	497	ctcaattgactgacggtag	48	207
114941	Coding	498	gctcaattgactgacggta	39	208
114940	Coding	499	tgctcaattgactgacgggt	23	219
114939	Stop	521	ggctcagcatttaggcagcca	18	210
114938	Stop Codon	531	tctcagcaaaggctcagcat	42	211
114937	3'UTR	601	gctaggaggccctggctgga	52	212
114936	3'UTR	613	ctctaaagatcctgcttaggag	39	213
114935	3'UTR	627	accactgtctccttctctaa	35	214
114934	3'UTR	642	atccagttcaaaataccac	0	215
114933	3'UTR	649	atttgatatccagttcaaa	20	216
114932	3'UTR	666	aaagcaaaaccaaaaatatt	7	217
114931	3'UTR	683	agagaggtagccactttaaa	45	218
114930	3'UTR	688	accaaagagaggtagccact	44	219
114929	3'UTR	713	cgtcacaatagagcaaagcc	14	220
114970	3'UTR	721	taagtccacgtcacaataga	7	221
114928	3'UTR	741	ttcatcaactccttattgct	8	222
114927	3'UTR	756	agagaacactgtcccttcat	15	223
114969	3'UTR	786	acaggcaccccgaccccac	4	224
114926	3'UTR	801	gaaccaagacccctgcacagg	59	225
114925	3'UTR	812	tatcacaatcagaaccaaga	34	226
114924	3'UTR	834	cattagcagccctgtatgga	18	227
114923	3'UTR	856	aaccacacttacccatggc	52	228
114922	3'UTR	903	gtggtagaaaaactcatcag	64	229
114921	3'UTR	934	acttttcaagtgattttat	13	230

As shown in Table 4, SEQ ID NOs: 187, 188, 192, 193, 196, 197, 198, 199, 202, 205, 207, 208, 211, 212, 213, 214, 218, 219, 225, 226, 228 and 229 demonstrated at least 30% inhibition of mouse Survivin expression in this experiment and are therefore preferred.

In accordance with the present invention, a second series of oligonucleotides were designed to target different regions of the mouse Survivin RNA, using published sequences (GenBank accession number AA717921, incorporated herein as SEQ ID NO: 231). The oligonucleotides are shown in Table 5. ATtarget

site@ indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 5 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse Survivin mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

TABLE 5
Inhibition of mouse Survivin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SITE	SEQUENCE	% INHIBITION	SEQ ID NO
114920	5'UTR	2	aatcccagccaaggatccga	0	232
114919	5'UTR	21	cgtggtggtcacaccttta	1	233
114918	5'UTR	33	tttcaagccggcggtgg	11	234
114917	5'UTR	57	acatatatatataaaacat	0	235
114916	5'UTR	87	aattttccttccttgatttt	5	236
114915	5'UTR	105	tactgagctacaaaactggaa	41	237
114914	5'UTR	108	acttactgagctacaaaactg	0	238
114913	5'UTR	168	aagtttattttttgtattt	0	239
114912	5'UTR	169	aaagtttattttttgtattt	7	240
114911	5'UTR	184	taaatcattaaaaggaaagt	0	241
114910	5'UTR	197	catcggtggcaagataaatca	0	242
114909	5'UTR	229	gcctgtccagggtgagatgc	0	243
114908	5'UTR	231	ttgcctgtccagggtgagat	0	244
114907	5'UTR	240	gggcaggcttgccctgtcca	13	245
114906	Start	293	ggtctccttgcctgaaatg	23	246
114905	Start Codon	296	gttgggtctccttgcctgga	59	247

As shown in Table 5, SEQ ID NOs: 237 and 247 demonstrated at least 30% inhibition of mouse Survivin expression in this experiment and are therefore preferred.

5

Example 19**Western blot analysis of Survivin protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 hours 10 after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 μ l/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to Survivin 15 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGERTM (Molecular Dynamics, Sunnyvale, CA).

20 **Example 20****Effect of antisense inhibition of Survivin on apoptosis**

ISIS 23722 and a mismatch control, ISIS 28598 (TAAGCTGTTCTATGTGTT; SEQ ID NO: 248) were assayed for their effect on apoptosis in HeLa cells. The caspase inhibitor z-VAD.fmk was purchased from Calbiochem (La Jolla, CA) and used according to manufacturer's recommendations. In HeLa cells without oligonucleotide, approximately 4% of cells are hypodiploid (indicating DNA fragmentation, a measure of apoptosis). With the addition of ISIS 23722, approximately 22% 25 of cells are hypodiploid, compared to approximately 11% with the mismatch oligonucleotide. In the presence of the caspase inhibitor z-VAD.fmk (42.8 mM), the percent of hypodiploid (apoptotic) cells drops to 3% without oligonucleotide, 6% with 30 ISIS 23722 and 4% with the mismatch control. This demonstrates

that antisense inhibition of Survivin increases apoptosis and that this effect is caspase-mediated.

Example 21**5 Effect of antisense inhibition of Survivin on cytokinesis**

HeLa cells treated with an antisense oligonucleotide targeted to Survivin (ISIS 23722) can be observed to form large, multinucleated cells as a result of improper cell division. The mismatch control oligonucleotide did not have 10 this effect and cells appeared normal (comparable to untreated controls). This effect can be quantitated by flow cytometry.

Untreated cells or cells treated with the control oligonucleotide display two prominent peaks, representing populations of cells in the G1 phase and the G2/M phase of 15 cell division, respectively. G1 cells have a single copy of their DNA (1x) and G2/M cells have two copies (2x). Over time from 24 hours to 72 hours, these 1x and 2x peaks remain virtually unchanged in cells treated with the control oligonucleotide or without oligonucleotide. However, in cells 20 treated with the antisense oligonucleotide targeted to Survivin, the majority of cells have two copies of DNA by 24 hours after oligo treatment. This indicates that cell division is arrested. By 48 hours after treatment with this oligonucleotide, a 4x peak is approximately equal in size to 25 the 1x and 2x peaks, indicating roughly equal numbers of cells with one, two and four copies of DNA. By 72 hours the largest peak is 16x, indicating that cells have 16 copies of their DNA and thus that division of the cytoplasm has not occurred for multiple generations. Thus inhibition of Survivin is shown to 30 interfere with cytokinesis.

Example 22**Effect of antisense inhibition of Survivin on cell proliferation**

Human HT1080 fibrosarcoma cells (American Type Culture Collection, CCL-121) were grown in minimal essential medium with 1% non-essential amino acids, 90% with 10% fetal bovine serum (Gibco BRL). Cells were electroporated (Electro Square 5 Porator, Model T820, Biotechnologies and Experimental Research, BTX) with oligonucleotide at settings of 225 volts for 6 milliseconds with a single pulse and oligonucleotide concentrations of 1 to 30 Φ M. ISIS 23722 (SEQ ID NO: 87) and the mismatch control ISIS 28598 (SEQ ID NO: 248) were used. 10 Cells were plated at 1500 cells/well immediately after electroporation and viable cells were measured by MTT assay at 24, 48, 72, 96 and 120 hours after electroporation. Growth rate (OD/hour) was plotted against oligonucleotide concentration. At an oligonucleotide concentration of 1 Φ M, 15 growth rates were virtually identical for ISIS 23722 and the control, ISIS 28598 (0.01726 and 0.01683, respectively. At 5 Φ M oligonucleotide, the growth rate of the ISIS 23722-treated cells was 16.7% less than the control treated cells (0.01433 vs. 0.01728 ?OD/hour, respectively). At 10 Φ M the growth rate 20 of the ISIS 23722-treated cells was 45% less than the control treated cells (0.009677 vs. 0.01762 ?OD/hour, respectively). At 20 Φ M the growth rate of the ISIS 23722-treated cells was 52% less than the control treated cells (0.007716 vs. 0.01620 OD/hour, respectively). At 30 Φ M the growth rate of the ISIS 25 23722-treated cells was 54% less than the control treated cells (0.006562 vs. 0.01417 OD/hour, respectively). Thus treatment with antisense oligonucleotide targeted to Survivin was demonstrated to reduce the rate of tumor cell proliferation by over 50%.

30 In an similar experiment using a different control oligonucleotide, a 20mer random oligonucleotide (ISIS 29848, SEQ ID NO: 249; NNNNNNNNNNNNNNNNNNN, wherein each N is a mixture of A, C, G and T) a similar result was obtained.

Oligonucleotides were tested at concentrations of 0.5 to 20 μ M, and cell viability was again measured by MTT assay and growth rate (OD/hour) was calculated. At 0.5 μ M oligonucleotide concentrations, growth rates were similar for 5 ISIS 23722 and control treated cells (0.01441 and 0.01342, respectively). At 10 μ M the growth rate of the ISIS 23722-treated cells was 57% less than the control treated cells (0.005568 vs. 0.01298 OD/hour, respectively). At 20 μ M the growth rate of the ISIS 23722-treated cells was 77% less than 10 the control treated cells (0.002433 vs. 0.01073 OD/hour, respectively). Thus treatment with antisense oligonucleotide targeted to Survivin was demonstrated to reduce the rate of tumor cell proliferation by over 75% compared to control.

A similar experiment was conducted in human MCF-7 breast 15 carcinoma cells, testing ISIS 23722 and the random control ISIS 29848 at doses from 0.5 to 20 μ M. Cells were electroporated (Electro Square Porator, Model T820 manufactured by Biotechnologies and Experimental Research, BTX) at settings of 175 volts for 6 milliseconds with a single 20 pulse with oligonucleotide and growth rates were calculated as described above. At 0.5 μ M oligonucleotide concentrations, growth rates were similar for ISIS 23722 and control treated cells (0.005959 and 0.005720, respectively). At 1 μ M oligonucleotide, growth rates were still relatively similar 25 for ISIS 23722 and control treated cells (0.005938 and 0.005479, respectively). At 5 μ M oligonucleotide, growth rates were 0.002574 and 0.005676, respectively for ISIS 23722 and control treated cells. At 10 μ M the growth rate of the ISIS 23722-treated cells was 69% less than the control treated 30 cells (0.001828 vs. 0.005901 OD/hour, respectively). At 20 μ M the growth rate of the ISIS 23722-treated cells was 64% less than the control treated cells (0.001523 vs. 0.004223 OD/hour,

respectively). Thus treatment with antisense oligonucleotide targeted to Survivin was demonstrated to significantly reduce the rate of tumor cell proliferation in several tumor cell types.

5

Example 23**Sensitization of cells to chemotherapeutic agent stimuli by ISIS 23722**

ISIS 23722 (SEQ ID NO: 87) and a control oligonucleotide, 10 ISIS 29848, a 20mer random oligonucleotide (ISIS 29848, SEQ ID NO: 249; NNNNNNNNNNNNNNNNNNN, wherein each N is a mixture of A, C, G and T) were assayed for their ability to sensitize cells to the effects of the chemotherapeutic agents, Taxol and Cisplatin.

15 Human HT1080 fibrosarcoma cells (American Type Culture Collection, CCL-121) were grown in minimal essential medium with 1% non-essential amino acids, 90% with 10% fetal bovine serum (Gibco BRL). Cells were treated with oligonucleotide at concentrations of 10 to 100 nM alone or in combination with 20 Taxol (concentrations of 0.25 nM or 1 nM) or Cisplatin (concentrations of 5 Φ M or 25 Φ M). Treatment with Taxol or Cisplatin followed oligonucleotide treatment by 1-2 hr. Cells were plated at 1500 cells/well immediately after treatment and 25 viable cells were measured by MTT assay at 12, 24, 36, 48, and 60 hours after treatment. Growth rate (OD/hour) is plotted against oligonucleotide and/or chemotherapeutic agent concentration.

A similar experiment was conducted in human MCF-7 breast carcinoma cells (American Type Culture Collection), 30 testing ISIS 23722 and the random control ISIS 29848 at doses from 10 to 100 nM alone or in combination with Taxol (concentrations of 0.5 nM or 2nM) or Cisplatin (concentrations of 2.5 Φ M or 15 Φ M). Cells were grown in Dulbecco's Modified Eagles medium. (low glucose), 90% with 10% fetal bovine serum

(Gibco BRL). Treatment with Taxol or Cisplatin followed 5 oligonucleotide treatment by 1-2 hr. Cells were plated at 2500 cells/well immediately after transfection and viable cells were measured by MTT assay at 12, 24, 36, 48, and 60 hours after treatment. Growth rate (OD/hour) is plotted against oligonucleotide and/or chemotherapeutic agent concentration.

Example 24**Mixed backbone version of active oligonucleotide ISIS 23722**

10 An oligonucleotide having the same sequence as ISIS 23722 (SEQ ID NO:87) was synthesized, this time as a 2' MOE gapmer with phosphodiester backbone linkages in the 2' MOE "wings" and phosphorothioate linkages in the 2' deoxy "gap". Both cytosines are 5-methylcytosines.

15 This compound is tested for its effects on cell proliferation, cytokinesis and sensitization to chemotherapeutic agents as described herein in previous examples.

Example 25**Down-regulation of IL-11-induced survivin expression on human skin engrafted onto immunodeficient mice**

25 The efficacy of anti-survivin antisense oligonucleotide cream to down-regulate IL-11 induced survivin expression in human skin grafts was examined in immunodeficient mice. SCID/beige mice were engrafted with human skin obtained from elective surgery and allowed to heal for 5 weeks. Four mice with 2 skin grafts each were used. The right graft of each animal received control (placebo) antisense oligonucleotide cream, while the left graft received the anti-survivin antisense oligonucleotide cream which comprised 5% of the 2--MOE mixed backbone deoxyoligonucleotide ISIS, 28599 (5--TGTGCTATTCTGTGAATT-3=, SEQ ID NO: 250) (nucleotides 1-4 and 15-18 are 2--MOE; cytosines at positions 5 and 10 are 5-meC;

nucleotides 1-3 and 16-18 are phosphodiester linkages; nucleotides 4-15 are phosphorothioate linkages. The dosing schedule consisted of regular dosing of 100 μ l/graft 3 times each day for 3 days. The morning of the 4th day, skin grafts 5 were given their morning dose then 1 hour later injected intradermally with recombinant human IL-11 (500 ng) (Genetics Institute, Andover, MA). Antisense oligonucleotide cream was administered for 2 final doses. Animals were sacrificed on the morning of day 5. Grafts were harvested and formalin 10 fixed for paraffin embedding.

Grafts from one animal were not identified in the blocks. The remaining three animals were evaluated by immunostaining for survivin and by routine hematoxylin and eosin histology. Immunostaining was performed on deparaffinized sections as 15 previously described. Briefly, 5- μ m tissue sections were cut and slides were baked overnight in a 60EC oven. Sections were deparaffinized in xylene for 5 hours, washed twice in ethanol, and endogenous peroxidase activity was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Slides were then 20 placed in a 4-quart Wear-Ever pressure cooker (Mirro Co., Manitowoc, WI) containing 1.5 liters of 9 mM sodium citrate, pH 6.0 that had been brought to a boil. The lid was secured, and heating was continued (approx. 6 min) until the pressure valve released. Slides were gently cooled by filling the 25 pressure cooker with tap water, and then washed 3 times with water and once with PBS, pH 7.0. Staining was performed using a primary rabbit polyclonal antibody against survivin at a concentration of 0.1 μ g/ml in PBS, pH 7.0, containing 0.5% bovine serum albumin (BSA) and 5% normal goat serum (Vector 30 laboratories) and incubated overnight at 4EC using a Histostain-Plus kit (Zymed) with 3,3'--diaminobenzidine (DAB) as the chromogen.

The histology appeared normal and indistinguishable

between the treated and control groups. Survivin expression showed a downregulation of expression in grafts treated with the survivin antisense oligonucleotide cream compared to controls. Both the dermal microvessels and the epidermal 5 keratinocytes showed this downregulation when compared to control grafts.

Example 26**Design and screening of duplexed antisense compounds targeting survivin**

10 In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target survivin. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion 15 of an oligonucleotide to survivin as described herein. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand 20 and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

For example, a duplex comprising an antisense strand 25 having the sequence CGAGAGGCAGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

	cgagaggcggacgggaccgTT	Antisense Strand
30	TTgctctccgcctgccctggc	Complement

RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacaon Research Inc., (Lafayette, CO). Once synthesized, the 35 complementary strands are annealed. The single strands are

aliquoted and diluted to a concentration of 50 uM. Once
 diluted, 30 uL of each strand is combined with 15uL of a 5X
 solution of annealing buffer. The final concentration of
 said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH
 5 7.4, and 2mM magnesium acetate. The final volume is 75 uL.
 This solution is incubated for 1 minute at 90°C and then
 centrifuged for 15 seconds. The tube is allowed to sit for
 1 hour at 37°C at which time the dsRNA duplexes are used in
 experimentation. The final concentration of the dsRNA
 10 duplex is 20 uM. This solution can be stored frozen (-
 20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are
 evaluated for their ability to modulate survivin expression
 according to the protocols described herein.

15

Example 27**Design of phenotypic assays and *in vivo* studies for the use
 of survivin inhibitors****Phenotypic assays**

20 Once survivin inhibitors have been identified by the
 methods disclosed herein, the compounds are further
 investigated in one or more phenotypic assays, each having
 measurable endpoints predictive of efficacy in the
 treatment of a particular disease state or condition.
 25 Phenotypic assays, kits and reagents for their use are well
 known to those skilled in the art and are herein used to
 investigate the role and/or association of survivin in
 health and disease. Representative phenotypic assays, which
 can be purchased from any one of several commercial
 30 vendors, include those for determining cell viability,
 cytotoxicity, proliferation or cell survival (Molecular
 Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based
 assays including enzymatic assays (Panvera, LLC, Madison,
 WI; BD Biosciences, Franklin Lakes, NJ; Oncogene Research

Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with survivin inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest. Analysis of the genotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the survivin inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.